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CELLULASE UTILIZATION RESEARCH

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PREAMBLE

Cellulase utilization research at SERI has taken a bidentate approach whereby multiple projects, some applied and some more background or basic in nature, are conducted concurrently. The applied projects are those which may directly lead to process methods, products or microorganisms capable of impacting immediately the economic picture of enzymatic conversion of biomass to liquid fuels. For FY1985-86 these applied projects include the evaluation of spray drying methods for cellulase preservation, optimization of the growth and cellulase enzyme production from a new SERI isolated aerobic thermophile, Acidothermus cellulolyticus, and the isolation of overproduced xylose isomerase from genetically modified E. coli (shown in "Overexpression of Xylose Isomerase" paper). Background studies conducted in FY1985-86 include the development of IMP/HPLC methods for the determination of the anomeric configuration of products from hydrolase action and the preparation of two textbook chapters on subjects in basic enzymology. The following paper consists of three sections describing these results.

I. EVALUATION OF SPRAY DRYING METHODS FOR CELLULASE PRESERVATION

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ABSTRACT

Spray drying processes are widely used for the large-scale preservation of biological goods. The application of this technology to the enhancement of the storability of cellulase and related enzymes would improve fermentation processes requiring input of specific quantities of enzyme catalyst of known activity. The present study found that very high (>90%) filter paper and carboxymethylcellulose hydrolyzing activities can be recovered from conventional spray dryers when operated at inlet air temperatures ranging from 90 to 180°C. The beta-

glucosidase and beta-xylosidase activity recoveries were somewhat lower and were optimal at temperatures ranging from 120 to 150°C. The individual performance of the 12 enzyme preparations studied was quite specific, however. An ultrasonic nozzle adaptation to the Yamato dryer demonstrated the overall feasibility of high energy atomization, inasmuch as the filter paper activity was retained after drying. The more labile beta-xylosidase activity was, however, entirely lost.

INTRODUCTION

Successful operation of industrial-scale fermentation plants based on enzymatic saccharification of biomass substrates demands the availability of appropriate quantities of saccharifying enzyme of known activity. Even with some redundancy in enzyme preparation facilities, losses of enzyme due to contamination or equipment failure can result in interruption of continuous operation. Access to stored and stabilized enzyme is clearly desirable.

Although some laboratory methods useful for the stabilization of enzyme preparations through dehydration (lyophilization, for instance [1]) have been successfully scaled up, spray drying is usually considered the most appropriate approach to large scale installations [2]. Unfortunately, the literature on conventional spray drying of biological materials has been largely concerned with the preservation and concentration of foodstuffs or food by-products. Some examples are cheese whey fractions [3,4], fish waste products [5], blood plasma proteins [6], bean protein [7], fungal mycelial fermentation broth [8], egg white [9], and many others. Studies describing the optimization of drying conditions for the successful spray drying of active enzymes are few, although those available include studies on fungal proteases [10], ribonuclease from Bacillus subtilis [11], milk additive enzymes (alkaline phosphatase) [12] and β-galactosidase from Aspergillus species [13]. Those specifically targeting cellulolytic and hemicellulolytic enzymes are not readily found in the literature.

In this study, preparations of commercially available enzymes and several cellulolytic enzyme-containing fermentation broths were subjected to bench-scale spray drying using the Yamato model GA-31 dryer. Enzyme activity recovery studies of dried products included filter paper activity, activity on carboxymethylcellulose (endocellulase), β-glucosidase and β-xylosidase activities as well as starch degrading ability (for amyloglucosidase). These parameters were evaluated as a function of the drying conditions (inlet and outlet temperatures) and sample preparation.

EXPERIMENTAL

Commercial enzyme preparations

The following cellulase preparations were studied: cellulase 150L from a selected strain of Trichoderma reesei, Genencor Inc.; Celluclast 1.5L (lot CNN3000) from T. reesei, NOVO Ltd.; cellulase C-0901 (lot 54F-0241) from P. funiculosum, Sigma Chem. Co.; Cellulysin (lot 405843) from T. viride, Calbiochem; Maxazyme C1 (ref. 5756) from T. reesei, Gist-

Brocades USA, Inc.; and cellulase "Onozuka" RS from a mutant strain of T. viride, Yakult Honsha Co., Ltd. The β-glucosidase enzymes G-8625 (lot 36F-4043) from almonds and Novozym 188 (batch DCN-01) from Aspergillis niger were obtained from Sigma Chem. Co. and NOVO Ltd., respectively. Cellobiase (batch DCN-01) from A. niger was also obtained from NOVO Ltd. Amyloglucosidase (lot 124F-0369) from Rhizopus genus mold was obtained from Sigma Chem. Co.

In-house cellulase enzyme preparations

Fourteen-liter fermentations of the Rut-C30 mutant of T. reesei were prepared according to the method of San Martin et al. [14] and 12-liter fermentations of Acidothermus cellulolyticus were grown according to Mohagheghi, et al. [15], for fresh enzyme spray drying. In both cases, the fermentation broth was freed of cells using a Cepa continuous centrifuge (30,000 rpm) and concentrated ten-fold at 4°C with a Millipore Pelicon ultraconcentrator equipped with a 10,000 M_r cutoff membrane cassette.

Spray drying equipment and operation

All spray drying studies were conducted on a Yamato model GA-31 bench scale dryer with electric heat control. Most drying tests were controlled at inlet temperatures of 90, 120, 150 or 180°C (corresponding to outlet temperatures of 30, 50, 65, 80, and 110°C), with filtered, compressed air (1.6 to 1.8 kg/cm^3) as the atomizer gas. The hot-air aspirator flow rate was controlled at 0.38 cfm. This setting was found to provide the optimum velocity for the cyclone collection system. Surface temperature thermocouples (Omega Engineering Inc., no. C03-K) were cemented on the collection cyclone to indicate enzyme powder contact temperature. On most runs, this was found to be 20°C lower than the indicated outlet temperature. A modified air distribution plate was fabricated to allow installation of a non-standard, ultrasonic nozzle (Heat Systems-Ultrasonics, Inc., Sonimist® nozzle # HSS-700-3). Operation of the ultrasonic nozzle required conversion to bottled nitrogen (98%) for the atomization gas and delivery to the nozzle at 60 psi. Samples were pumped to the dryer at a flow rate of 2 mL/min in 20-100 mL aliquots per run after adjustment to 5% w/v.

Analytical HPLC

Enzyme preparations were subjected to analysis by Ion Mediated Partition (IMP)/HPLC to determine possible monosaccharide and polysaccharide contents. IMP chromatography was also used to screen for preservatives, such as glycerol, sorbitol, ethylene glycol and others. The HPLC system used has been described earlier [16].

Chemical analysis of enzyme preparations

All enzyme preparations were analyzed for protein, reducing sugar and salt content. These are presented as weight-percent relative to starting material (usually powders). In the case of liquid enzyme preparations, the solids content of aliquots were determined by both lyophilization and oven drying at 60°C for 24 h. Protein content was

determined by the modified Lowry method of Markwell et al. [17] and by the dye binding method of Bradford [18]. Reducing sugars were determined by the alkaline-DNS (3,5-dinitro salicylic acid) method described by Miller [19]. Salt content was determined as conductance equivalence based on a NaCl series as standards. A Beckman model RC-16C conductivity bridge with a 1 cm cell was used for this determination.

Enzyme assays

Assays for cellobiase, endo-β-1,4 glucanase (CMCase), and saccharifying cellulase (FPU) activities followed the methods of Sternberg et al. [20] and Mandels et al. [21] as modified in a recent IUPAC report [22]. Here, one cellobiase unit equals that amount of enzyme converting 2.0 μmol glucose per minute from cellobiose, one CMCase unit equals that amount of enzyme liberating 1.0 μmol glucose (or reducing sugars as glucose) from carboxymethylcellulose per min, and one filter paper unit of activity equals that amount of enzyme complex yielding 1.0 μmol glucose per minute from 50 mg filter paper strips. Under the recommended conditions of the CMCase and filter paper activity assays, enzyme/substrate ratios must be adjusted so that 0.5 mg and 2.0 mg glucose is released per hour at 50°C, respectively. β-glucosidase was determined according to the method of Wood [23] as aryl-β-glucosidase by the hydrolysis of p-nitrophenyl-β-glucopyranoside (Sigma Chem. Co.). The concentration of p-nitrophenol was estimated from the extinction at 410 nm under alkaline conditions induced by the addition of 2 M Na_2CO_3. One unit of activity is defined as that amount of enzyme that catalyzes the cleavage of 1.0 μmol substrate per min. β-xylosidase activities were determined in a similar way using p-nitrophenyl-β-xylopyranoside as subsrate [24]. Glucoamylase activity was determined using soluble potato starch (Sigma Chem. Co.) as substrate by the method given in Tucker et al. [25]. Here one unit of activity is given as that amount of enzyme which releases 1.0 μmol of glucose per min from a 1% starch solution at 55°C.

Spray drying methods

The enzyme preparations available as dry powders were spray dried as a 5% w/v solution. The liquid preparations were diluted to give approximately this concentration after evaluating the solids content of the original sample. The NOVO liquid enzyme preparations required ultrafiltration/dialysis using an Amicon PM10 membrane to remove the glycerol (included as preservative by NOVO) and excess glucose before spray drying. Several spray drying adjuvants (0.5% w/v) were studied. These included fumed silica (Sigma Chem. Co.), maltodextrin® G-250 (Grain Processing Co.), 1.0 M NaCl, and maltose (Sigma Chem. Co.).

Enzyme powder storage and activity retention

Spray dried powders of Genencor cellulase 150L and NOVO Celluclast 1.5L were stored under similar conditions with filter paper and beta-glucosidase activity checked every two weeks for 2 months. Storage conditions were under nitrogen or air at 25°C.

Table I

Table I
SELECTED PROPERTIES OF ENZYMES SUBJECTED TO SPRAY DRYING

Enzyme type	Source	Solids[a] mg/mL	Protein content g Lowry protein /g preparation	Sugar content g sacchar-ides/g prep.	Salt content g NaCl equiv./g prep.	IFPU/g[b] Lowry protein	CMCU/g Lowry protein	βGU/g Lowry protein	βXU/g Lowry protein
cellulase	Genencor	165	0.196 (0.052)[c]	-0-	0.0036[c]	558	14,500	1470	22
cellulase	NOVO	487[49]	0.171 (0.042)[c]	0.004[c]	0.0064[c]	417	7,700	121	150
cellulase	Sigma	powder	0.50	0.022	0.190	583	10,800	8077	145
cellulase	Calbioch.	powder	0.37	0.50	0.066	325	13,100	1063	225
cellulase	Gist-Bro.	powder	0.29	0.744	0.039	565	12,800	660	466
cellulase	Rut-C30	44[d]	0.023 (0.001)[c]	-0-	0.012[c]	556	17,800	289	453
cellulase	A. cell.	56[d]	0.020 (0.001)[c]	-0-	0.0006[c]	127	650	30	4
cellulase	Yakult	powder	0.52	0.568	0.374	366	9,573	395	40
β-gluco.	Sigma	powder	0.94	-0-	0.133	NA[e]	NA	6500	NA
β-gluco.	NOVO	391[34]	0.140 (0.020)[c]	0.172[c]	0.055[c]	NA	NA	1789	NA
cellobiase	NOVO	397[44]	0.147 (0.020)[c]	0.167[c]	0.056[c]	NA	NA	1677 (14,490)[f]	NA
amylogluc.	Sigma	powder	0.42	0.133	0.053	19,788[g]	NA	NA	NA

[a]Determined by air drying at 60°C for 48 h. Spray drying of NOVO preparations was performed after samples were subjected to Amincon PM10 ultradialysis (values shown in square brackets only). [b]Enzyme activities are defined in text. [c]Components of liquid forms of enzyme are given as g/mL preparation (Note: obtain units/ml or units/gram of original preparation from the product of the protein content and specific activity). Parenthetical values from dye-binding protein assay illustrate possible protein assay-specific bias in specific activity data. [d]Spray dried as shown (represents a 10 fold concentration from fermentation broth). [e]NA is not analyzed. [f]Assayed as cellobiase activity (see text). [g]Assayed as starch degrading activity.

Table II
SPRAY DRYER NOZZLE COMPARISON USING GENENCOR CELLULASE 150L

Nozzle type	Drying Temperature °C	IFPU/g protein	CMCU/g protein	β-GU/g protein	β-XU/g protein
Standard[a]	150	528	13,639	1,450	19
ultrasonic[b]	150	465	12,750	409	0.70

[a]For purposes of comparison, data shown for standard nozzle were taken from Figures 1 - 4.
[b]The Sonimist® model 700-3 ultrasonic spray nozzle (Heat Systems-Ultrasonics, Inc.) was adapted to the Yamato GA-31 spray dryer by design of a new hot-air distributor plate.

RESULTS AND DISCUSSION

Table I illustrates many important characteristics of 10 commercial and 2 in-house hydrolytic enzyme preparations. Of the liquid cellulase preparations studied, the Genencor 150L enzyme had the highest overall filter paper activity (110 IFPU/mL preparation), with NOVO Celluclast 1.5L, Rut-C30 and A. cellulolyticus producing 71, 13 and 2.5 IFPU/mL, respectively. The Sigma cellulase appeared to have the highest activity (292 IFPU/g) of the powder preparations, with Yakult-Honsha RS, Gist-Brocades C1 and Calbiochem producing 190, 164 and 120 IFPU/g preparation, respectively. Also, the Sigma enzyme demonstrated the highest beta-glucosidase activity, even higher than the NOVO 188 preparation which is sold as beta-glucosidase. All enzymes showed relatively low, but measurable, beta-xylosidase activity. Note that these values are independent of Lowry protein content values given in Table I; however, activity values are given in Table I as units/gram Lowry protein (specific activity) to allow comparison with materials recovered from the spray dryer receiver and cyclone where a partial fractionation of components may occur. Table I also shows several important trends in cellulase content: the Sigma enzymes appear to contain a very high salt content; the Gist-Brocades, Yakult-Honsha and Calbiochem enzymes contain a very high sugar content (mostly glucose); and after diafiltration with an Amicon PM10 membrane, the NOVO preparations lose more than 90% solids (HPLC analysis shows that half of this is glycerol and glucose).

The characteristics described above proved important in interpreting spray drying performance. The NOVO preparations required diafiltration to remove high concentrations of glycerol added as preservative before spray drying was possible. The following conditions were found to give good collection of dried product: sample at a concentration of 5% w/v, aspirator rate of 0.38 to 0.40 cfm, inlet air temperature of 120 to 150 °C and a sugar content below 0.5% w/v. Adjuvants added to samples before spray drying included fumed silica, maltodextrin®, salts and monosaccharides. No adjuvant examined significantly improved the poor performance of problem preparations (i.e., product burning or sticking to glassware); however, addition of fumed silica was found to produce very low beta-glucosidase activity recoveries while having no effect on filter paper activity recovery.

Most cellulase preparations examined showed IFPU/g and CMCU/g recoveries of 90 to 100% in the inlet air temperature range 90 to 180°C. The beta-glucosidase and beta-xylosidase activity recoveries were high only in the 120 to 150°C range, with many beta-xylosidases showing poor activity recovery under all conditions. The amyloglucosidase examined showed very good activity recovery (95%) in the range 120 to 150°C. These activity recoveries are somewhat higher than those found for Aspergillus species β-galactosidase (66%, [13]) and Saccharomyces fragilis β-galactosidase (86%, [26]).

Studies of enzyme activity retention upon storage of spray dried preparations showed that NOVO Celluclast 1.5L retained nearly 100% of initial filter paper and beta-glucosidase activities after two months at 25°C under air, regardless of spray drying temperatures used (which
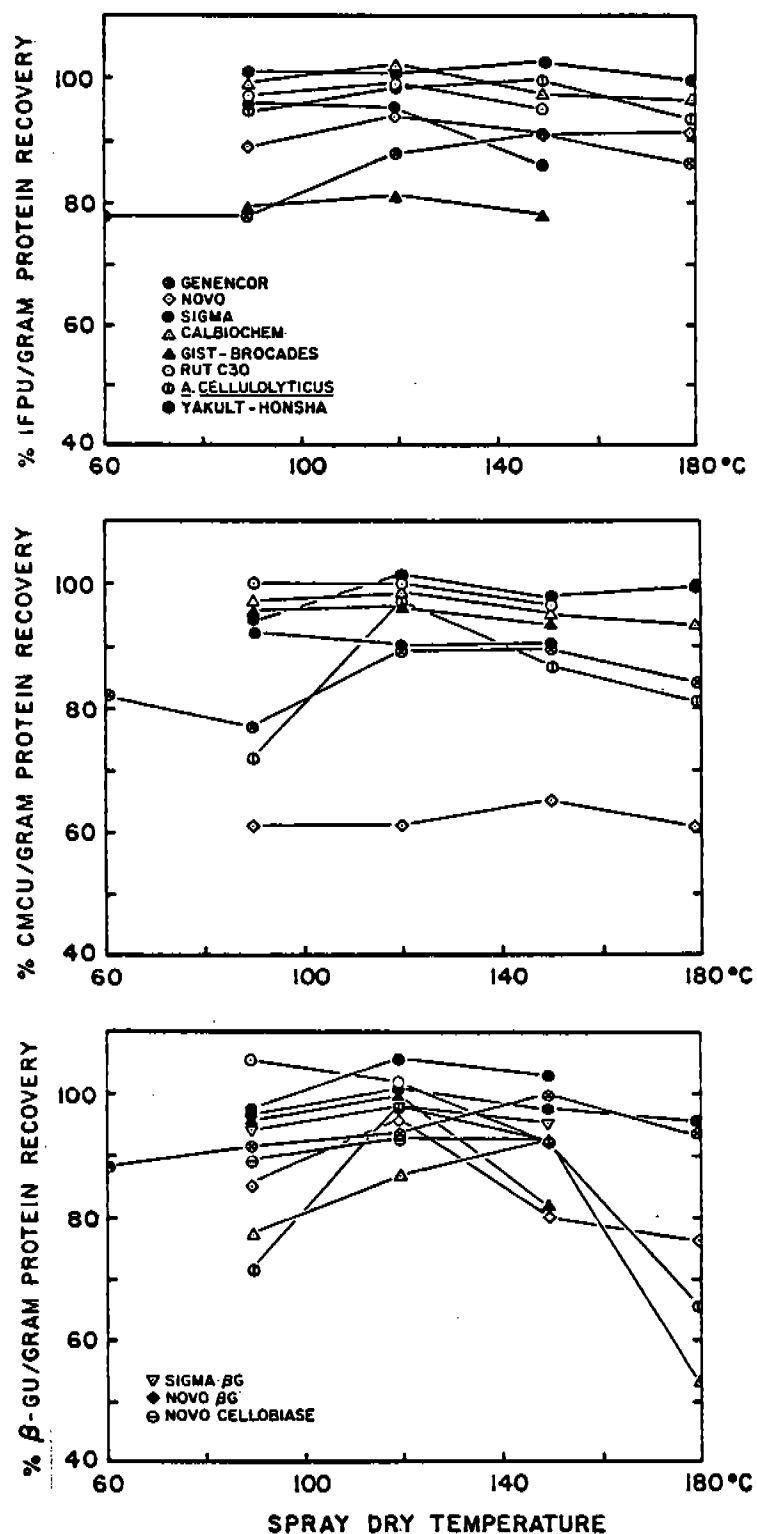
Figure 1. Comparison of the percentage of international filter paper (IFP), carboxymethylcellulase (CMC), and beta-glucosidase (β-G) units per gram protein recovered from starting material plotted at various inlet air temperatures for different cellulase preparations.
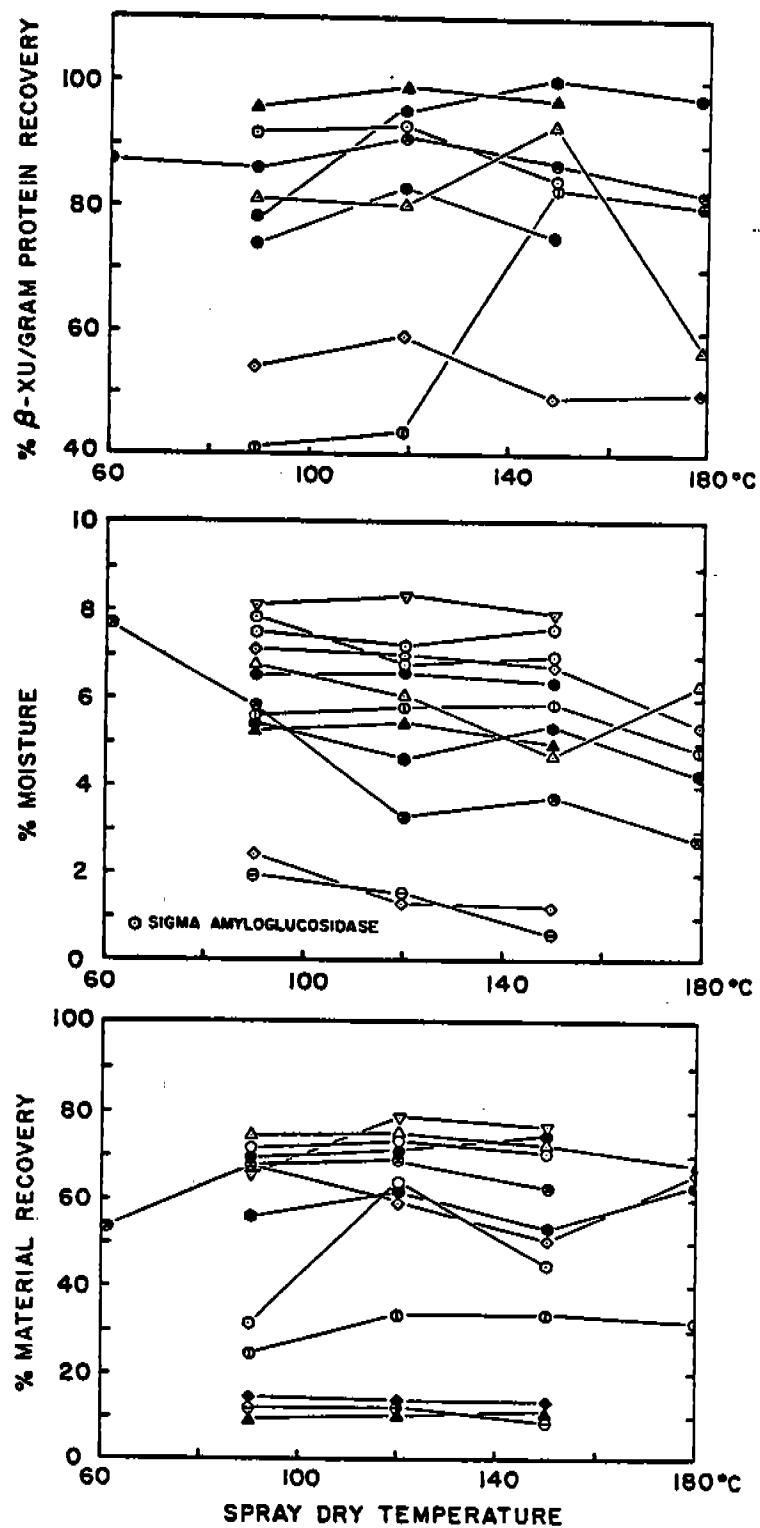
Figure 2. Comparison of the percentage of beta-xylosidase (β-X) units per gram, moisture and material recovered from starting material plotted at various inlet air temperatures for different cellulase preparations.

influences the moisture content of the powder). Early results showed that storage under nitrogen instead of air had no effect. The Genencor cellulase 150 L, however, demonstrated a 60% loss in activity under similar storage conditions. A possible explanation for this difference may lie in the trace quantities of glycerol entrained in the NOVO powders after spray drying.

The ultrasonic nozzle adaptation to the Yamato dryer provided some insight into possible ultrasonic drying of cellulase component enzymes. Table II shows that the high energy nozzle produced no effect on the recovery of overall filter paper activity, but had a devastating effect on the beta-glucosidase and beta-xylosidase activities. This observation is supported by the well known relative instability in solution of these classes of enzymes.

FUTURE DIRECTION

The cellulase spray drying research is now completed and the methodology will be used for the preparation of enzymes used in-house.

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IIa. ACIDOTHERMUS CELLULOLYTICUS:
A NEW AEROBIC CELLULOLYTIC THERMOPHILE

Ali Mohagheghi, Karel Grohmann, and Michael E. Himmel

SUMMARY OF CHARACTERISTICS
(an excerpt from Mohagheghi, et al. [1])

Twelve isolates of thermophilic, acidophilic, cellulolytic bacteria were obtained from three different primary enrichment cultures from acidic

hot springs at Yellowstone National Park. The three isolates that had the highest cellulolytic activity, as shown by the diameter of clearing zones surrounding colonies on cellulose agar plates, were selected for intensive study. All were gram-variable, nonsporulating aerobic rods which formed no pigment. The guanine-plus-cytosine content of the deoxyribonucleic acid was 60.7 ± 0.6%. The organisms are resistant to penicillin G at 100 µg/ml. They share several important features with strains of Thermus, namely heterotrophic, aerobic, thermophilic mode of growth; morphological features; sensitivity to lysozyme; and presence of catalase. They differ in other important aspects, such as the pattern of carbon sources utilized for growth, pH-and temperature-profiles of growth, the pattern of sensitivity to antibiotics, the guanine plus cytosine content of DNA, the composition of amino acids in the cell walls, and the structure of the cell walls. Thermus species are very sensitive to penicillin G while our strains are resistant. Our strains also are different in important respects from the genus Thermomicrobium. We therefore designate the organism as the new genus, new species, Acidothermus cellulolyticus, gen. nov., sp. nov. of which the type strain is our 11B, ATCC 43068.

IIb. ACIDOTHERMUS CELLULOLYTICUS:
OPTIMIZATION OF CELL GROWTH AND EVALUATION OF ENZYME PROPERTIES

Melvin P. Tucker, Ali Mohagheghi, Michael E. Himmel and Karel Grohmann

ABSTRACT

The microorganism was found to grow at 37 to 65°C with an optimum of 55 °C. The pH range for growth was 3.5 to 7.0 with an optimum of 5. Cell densities of 14 optical density units were easily obtained by adjustment of Wolin salts and LPBM (low phosphate basal medium) concentrations in growth media and maintainance of high oxygen tension levels (from 70 to 95 % saturation). The supernatant cellulase enzyme complex was found to have a temperature optimum of 75 °C and appears to be relatively insensitive to cellobiose inhibition (60% retention of filter paper activity in the presence of 10 mM cellobiose). Preliminary enzyme purification studies indicate that the filter paper activity producing complex is composed of components smaller than 150,000 daltons.

INTRODUCTION

The driving hypothesis for this research is that a thermophilic, acidophilic, cellulolytic bacterium could be grown in coculture with other thermotolerant microorganisms to produce ethanol from cellulosic wastes at a high rate. Alternatively, the cellulolytic organism could be used to produce cellulase which could be added to a cellulose-containing culture of an organism which ferments glucose to ethanol. Most of the thermophilic, cellulolytic microorganisms isolated to date are spore-forming anaerobes belonging to the genus Clostridium. None of these are acidophilic, all having an optimum pH near 7. Thus they would not grow well under the acidic conditions (pH 3 to 5) prevalent in an ethanolic fermentation.

Aerobic, thermophilic, cellulolytic microorganisms include several species of fungi [2] and a few species of filamentous bacteria belonging to the family Actinomycetaceae [3]. Among the fungi, Myceliophthora thermophila is of considerable interest, since it grows well on cellulose in submerged culture at 50°C and pH 4.5, and synthesizes cellulolytic enzymes [2]. Thus the organism is acidophilic, as well as cellulolytic and thermophilic. Rosenberg [4] screened 21 species of thermophilic and thermotolerant fungi for the ability to degrade cellulose, and found that 13 species had this ability. It was not determined whether these organisms could grow well in submerged culture under thermophilic and acidophilic conditions and produce significant amounts of cellulase. Aspergillus fumigatus has been shown to grow well on cellulose in submerged culture at 45°C and pH 4.6, and to produce considerable amounts of cellulase [5].

Among the known thermophilic, aerobic bacteria, only a few actinomycetes are actively cellulolytic, notably Thermomonospora curvata [6] and Thermoactinomyces cellulosae [7,8]. However, neither of these organisms is acidophilic. The acidic hot springs at Yellowstone National Park provide an excellent source for the isolation of acidophilic thermophilic bacteria. The distribution of pH among the springs at Yellowstone is bimodal, with a large group in the highly acidic range, pH 2-5, and another group in the near-neutral to alkaline range, pH 6-10. Brock [9] isolated Thermus aquaticus, Bacillus acidocaldarius, and Sulfolobus species from Yellowstone springs, and Jackson et al. [10] isolated thermomicrobium. All of these organisms are obligate thermophiles, and Bacillus acidocaldarius and Sulfolobus are acidophilic, but none of these are known to be cellulolytic.

EXPERIMENTAL

Bacterial Strains

The 12 isolates described were obtained from the upper Norris Geyser basin area in Yellowstone National Park under permit No. A9015 Yell. The acidic springs selected had temperatures of 45 to 65°C and a pH range of 4 to 5.5. All of the isolates formed similar colony types on cellulose agar and D-cellobiose agar, and all showed identical morphology. The type strain, 11B, has been deposited with the American Type Culture Collection (ATCC), Rockville, Md., under the number 43068. All strains have been successfully maintained both in a freeze-dried state and frozen at -70°C after addition of 77 µL dimethylsulfoxide (DMSO) per mL of culture suspension. Strain 11B was selected for further study because of high cellulose-clearing ability on cellulose-agar plates.

Effects of Temperature and pH on Growth

The relationships between growth rate, temperature, and pH were determined by chemostat studies in Biogen and Bioflo (New Brunswick) fermentors using 2-liter reactors charged with 1.2 liter of LPBM medium supplemented with 1 g/liter yeast extract and 10 g/liter D-cellobiose.

Storage of Strain 11B

A. cellulolyticus strain 11B was carried by growth on agar plates with phosphate-swollen cellulose as substrate. The strain was subcultured on a weekly to biweekly basis and growth on plates was used as inocula for experiments.

Fermenter Studies

In fermentation studies for high growth densities, and for cellulase production, a New Brunswick Microferm 14-liter fermenter or Braun Biostat S fermenter with 4- and 12-liter vessels were used. The fermenters were equipped with pH control and dissolved-oxygen monitoring. The Biostat S was also equipped with a dissolved-oxygen controller which used an electronic metering valve to regulate gas flow to the fermenter. In some experiments UHP oxygen (99.995%) was used in place of compressed air in order to maintain the dissolved oxygen at sufficient levels for the fermentation. Cell density (before cellulose addition) was followed by optical density (O.D.) measurements at 590 nm in a 1 cm cuvette using a Bausch and Lomb Spectronic 21.

Fermentation Inocula

A. cellulolyticus grown on phosphate-swollen cellulose plates was used to inoculate 50 mL of medium containing cellobiose (0.5%) as substrate in 250 mL shake flasks. The flasks were incubated in a 55 °C shaking incubator until turbid growth occurred (O.D. ~1.0) and then aseptically transferred to either 500 mL or 1 liter of medium in 2800 mL shake flasks. The shake flasks were again incubated until turbid growth ensued and used to inoculate the fermenter.

Measurements of Cellulase Activities

Measurements of cellulase as international filter paper (IFP), CMCase, beta-glucosidase and beta-xylosidase activities were performed as described in Section I of this chapter with the exception of the assay incubation temperature, which was 65 °C.

Concentration of Growth Supernatant

Concentration of cellulase enzyme activity from 12-liter fermentations was performed by first removing the cells and particulates from the fermentation broth using a Cepa continuous centrifuge. The centrifugation was performed at 30,000 rpm with the bowl controlled at ~4 °C. The clarified liquid was filtered using a Gelman pressure filtration unit with fine glass fiber filter (GFF). The filtrate was then concentrated ten-fold using a Millipore Pellicon cassette system equipped with 10,000 MW cutoff polysulfone membrane. The concentrate was filter-sterilized using the Gelman pressure filtration unit equipped with a presterilized 0.2 micron filter cartridge.

Cellulase Enzyme Purification

The ultraconcentrate from the Pellicon system was diafiltered against 10

mM acetate buffer pH 5.0 with 100 mM NaCl using an Amicon hollow fiber concentrator. After complete exchange, the sample was further concentrated (50 fold) and loaded on a size exclusion chromatography column measuring 5 x 90 cm (TSK HW55S). Activity was found only in the fractions eluting immediately after the void volume and well before the total column volume. These fractions were pooled, dialyzed against 10 mM acetate buffer (using an Amicon stirred cell concentrator with a PM10 membrane), and loaded zonally onto an ion-exchange column measuring 2.6 x 90 cm (TSK 650S). The column was washed with ten column volumes of loading buffer, and then eluted with a linear gradient consisting of one liter of 10 mM acetate buffer and one liter of 10 mM acetate buffer in 500 mM NaCl. These fractions were also assayed for the four types of activity discussed above.

RESULTS AND DISCUSSION

As a result of screening, several isolates of thermophilic, cellulolytic bacteria growing in the acidic pH range were obtained. The microorganisms we have isolated are a result of selective conditions applied during enrichment and isolation and do not represent major microbial populations in geothermal hot springs. The number of thermophilic microorganisms that were discarded during the isolation process far exceeds the number of isolates we have obtained. It should be noted, however, that no thermophilic fungi, yeasts, and actinomycetes were observed during enrichment and isolation. The majority of bacteria we observed during the initial stages of isolation were sporulating rods resembling bacteria of the genus Bacillus. A surprising result was a uniformity of cellulolytic strains we have isolated from various springs.

Improvements in the understanding of the nutritional requirements of Acidothermus cellulolyticus strain 11B have allowed faster growth rates and higher densities to be obtained. Preliminary information on the cellulase enzyme kinetics and end-product inhibition are described below.

Figure 1A shows the growth-temperature and pH optima of A. cellulolyticus grown in submerged culture. As predicted, the conditions of 55 °C and pH 5.5 found optimal for the isolated strain were identical to that of the Yellowstone spring from which it was taken. Interestingly, the filter paper activity found in the supernatant of the culture showed a temperature optimum of 75 °C, with nearly 50% of the highest activity remaining after one hour at 80 °C (see Figure 1B).

The growth of A. cellulolyticus strain 11B using LPBM medium supplemented with 0.5 g/liter yeast extract (Difco) has been demonstrated to have a long lag time and result in optical densities of ~4.0 (see Figure 2A). It was found that supplementing the normal medium with a trace mineral solution (Wolin, [11]) resulted in a shorter lag time with faster growth and higher densities. Increasing the medium to 2 x LPBM and 1.0 g/liter yeast extract to accommodate the rapid use of nutrients in high density fermentation runs resulted in densities of greater than 14 O.D. (see Figure 2B). In the fermentation run detailed
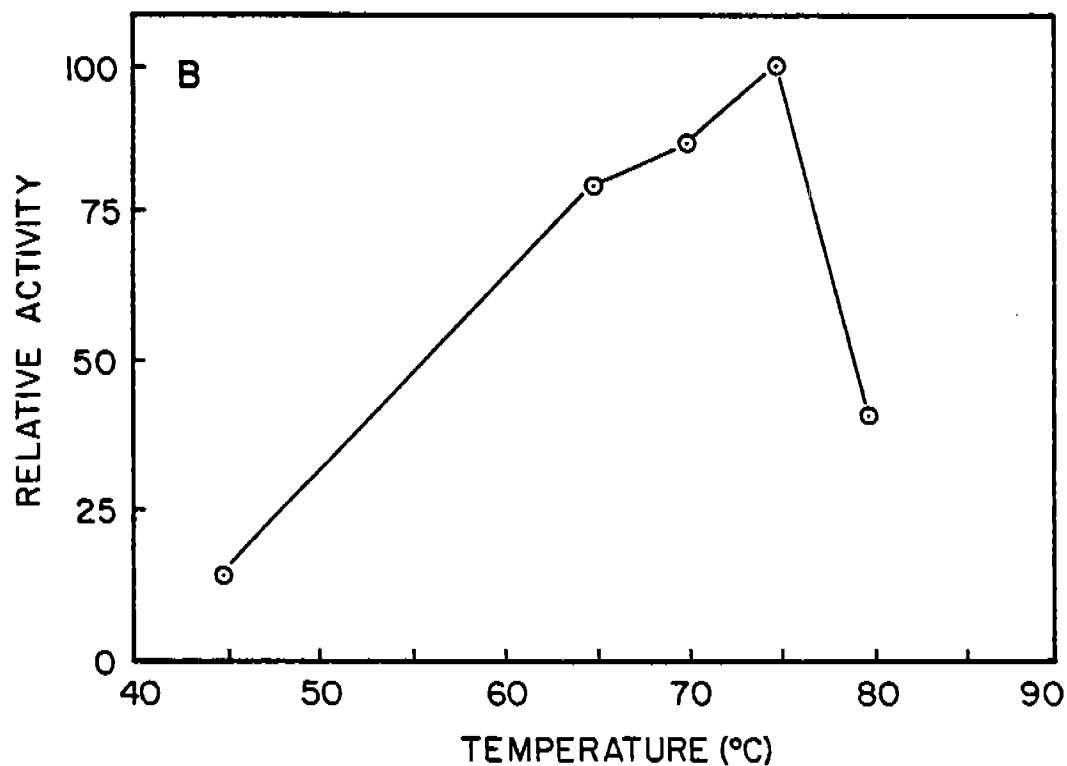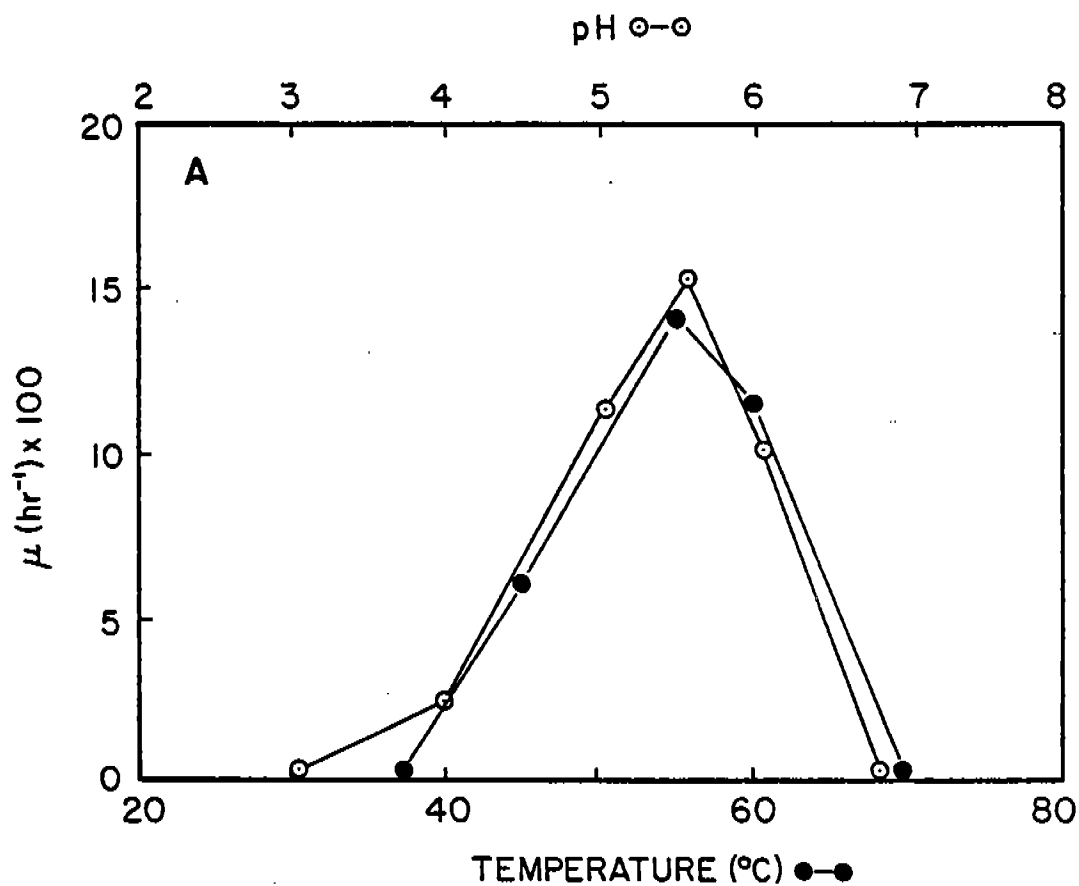
Figure 1.   (A) Plot of specific growth rates at differing temperatures
(held at pH 5.5) and differing pH values (held at 55 °C) for strain 11B.
(B) Filter paper activity of supernatant from 11B after incubation for one
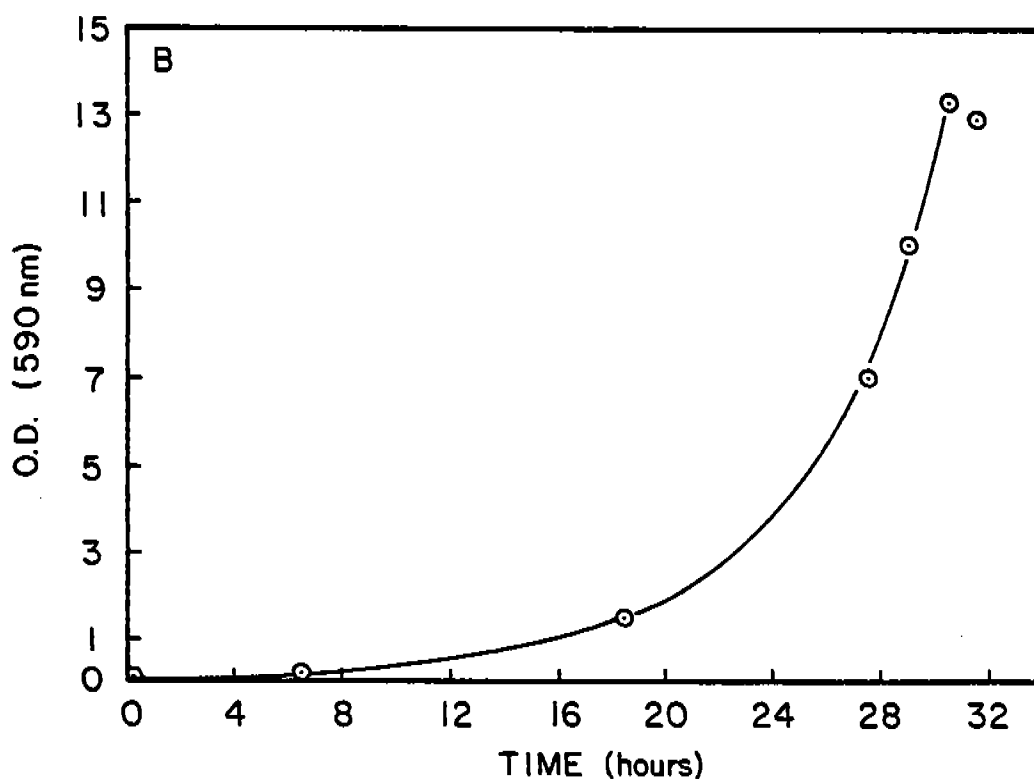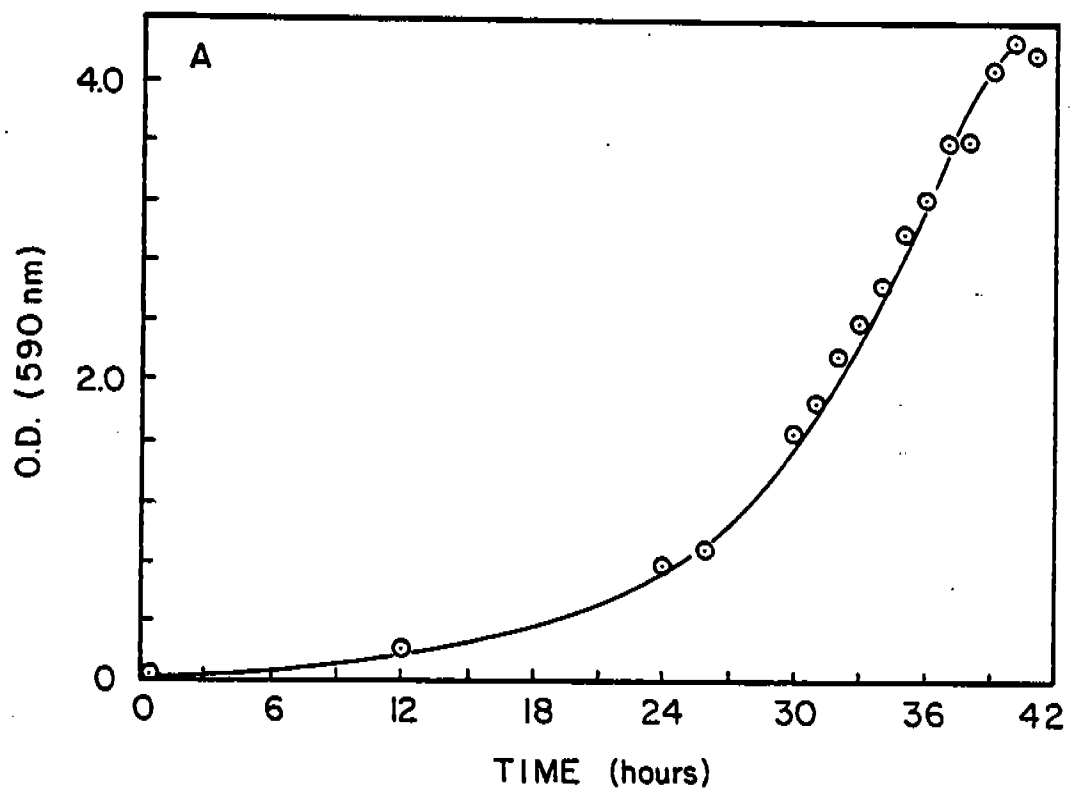hour at temperature shown.

Figure 2.   Growth curves of strain 11B before (A) and after (B) optimization of Wolin salts, LPBM concentration and oxygen control.

in Figure 2B, the growth was limited at 14.4 O.D. by substrate depletion.

During the growth of A. cellulolyticus 11B using the complete medium described above, it was discovered that the demand for oxygen at high cell densities appears to be critical. Changing to a type of sparger that allowed smaller bubbles restricted air flow and caused severe foaming problems. Although A. cellulolyticus tolerates antifoam agents in the growth medium, these agents were not used due to problems in down stream processing of the fermentation broth for cellulase enzyme activity. When culture densities reach an O.D. of 7-10, sparging of the fermenter with the maximum air flow of 14 liters per minute was not sufficient to meet the requirements of the culture. In this case, the dissolved oxygen became limiting and the viability of the culture was diminished. Even short periods of oxygen limitation (10-15 minutes) were sufficient to severely affect the fermentation. The growth of A. cellulolyticus at high densities was found to actively consume cellobiose (O.D. 12.4 used 0.5% cellobiose in 15 minutes) and if cellulose was present in the medium along with cellobiose, the microorganism required only a 1-3 hour lag phase after cellobiose exhaustion before vigorous growth on cellulose ensued. In comparison, the lag phase was longer (6-20 hours) when the cellulose was added just as the cellobiose in the fermenter was exhausted. Cellulase enzyme activity in fermentations using cellobiose as sole substrate was not significant (~0.02-0.05 IFPU/mL or less) whereas growth on 0.5% cellobiose with 1.0% cellulose (Solka floc SW200) resulted in 0.10 IFPU/mL after 30 hours (unfortunately, the culture became oxygen limited before extensive utilization of the cellulose occurred).

Figure 3 shows the results of cellobiose inhibition on the overall filter paper activity found in the concentrated supernatant from Acidothermus. Compared to the control (no cellobiose), cellobiose levels of 2 mM caused less than 10 % loss in apparent activity. Even cellobiose levels as high as 10 mM induced only a 40 % loss. These results compare very favorably with the 50% loss in activity on alpha-cellulose displayed by CBH from T. viride in the presence of 1.13 mM cellobiose [12].

The active enzyme components from the supernatant of A. cellulolyticus have been mapped preliminarily by size exclusion chromatography and ion-exchange chromatography (see Figures 4A, and 4B, respectively). Unlike cellulase systems studied from Clostridium thermocellum [13], the filter paper activity from the Acidothermus system does not elute in the very high molecular fraction found in the column void volume. Instead, all activities examined eluted in the 150,000 to 30,000 dalton range from the TSK column, with the filter paper activity associated with both the 150,000 dalton and 90,000 dalton peak and the beta-glucosidase activity associated with the 45,000 dalton peak. All activity was found to adsorb to the TSK 650S column and was eluted throughout the 0 to 0.5 M gradient applied to the column. Compartmentalization of four activities is clearly seen in Figure 4B, with filter paper activity occurring at points of multiple peak overlap.

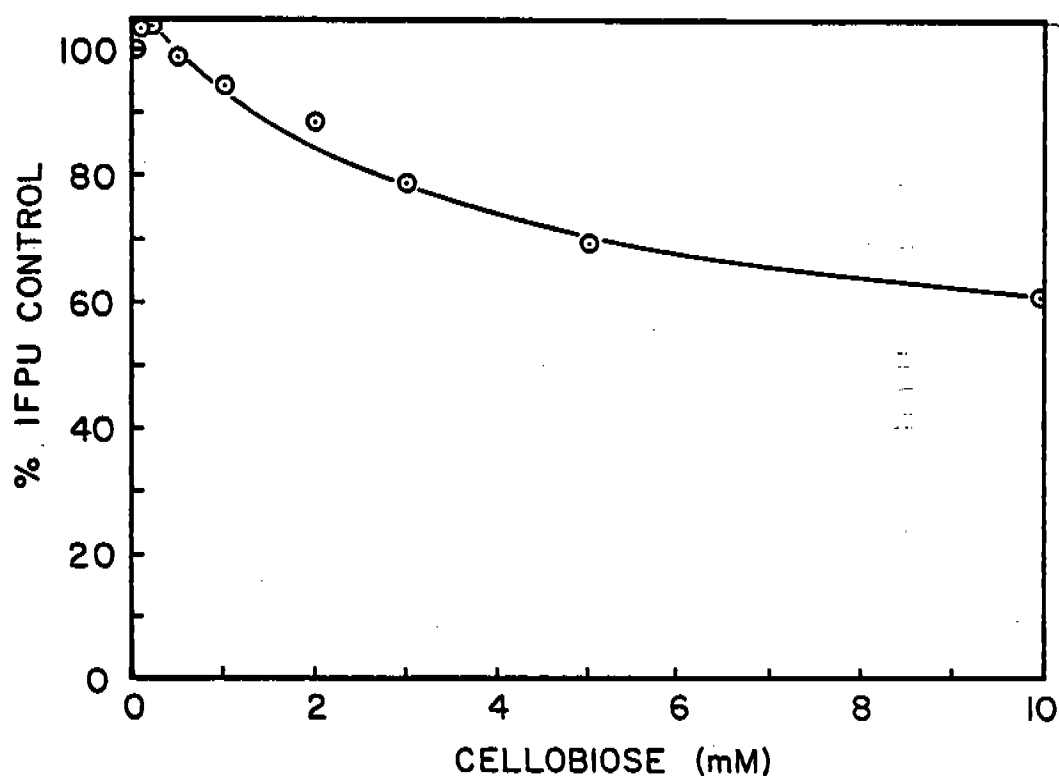Results from these initial attempts at optimization of the growth of

Figure 3. Study of cellobiose inhibition of cellulase activity in supernatant from A. cellulolyticus strain 11B. Supernatant was concentrated and dialyzed by hollow-fiber diafiltration (Amicon) with 10 mM acetate buffer pH 5.0. IFP assay incubation mixture included cellobiose at concentrations shown.

A

GGGGGG
XXX XXXXXX
F   F
V   V VVVVV

B

GGGGGGGGGGGGGG
XXXX
F FF FF FF FF   FFFFF
V   VV   V V   VV   VV

Figure 4. Results of preliminary fractionation of supernatant from 11B grown on cellobiose and induced on cellulose. (A) and (B) show the profiles obtained from size exclusion chromatography on TSK HW-55S followed by ion-exchange chromatography on TSK 650S, respectively. The abreviations F, G, X, and V, refer to filter paper, beta-glucosidase, beta-xylosidase and viscosity asssay activities.

Acidothermus cellulolyticus strain 11B are encouraging. The microbe can be grown to high cell densities (14 O.D. and greater) in 24 to 32 hours. Acidothermus produces a cellulase which is stable under extremes of pH and temperature. The present level of activities and productivities (0.12 IFP units/mg Lowry protein, 5.0 CMCase units/mg and 4 IFP units/liter/hour, repectively) from Acidothermus have not yet met those of Trichoderma reesei wild type QM6a (i.e., when grown on peptone-6% cotton, QM6a produces 0.70 IFP units/mg, 12 CMCase units/mg and 15 IFP units/liter/hour; when grown on cornsteep liquor-5% Solka floc, QM6a produces 0.52 IFP units/mg, 22.5 CMCase units/mg and 18 IFP units/liter/hour, respectively [14]). Also, the T. reesei mutant RUT C30 has been shown to produce filter paper specific activities of 1.22 IFP units/mg and productivities of 108 IFP units/liter/hour [14].

It is anticipated that the optimization of fermentation conditions, selection of chemical inducers, and mutant selection will in time result in great improvements in the performance of Acidothermus. When improved cellulase productivities are coupled to the fast growth rate and high cell densities already attainable, Acidothermus may well represent a valuable component in thermophilic, saccharification/fermentation processes, as well as providing a convenient model system for bacterial cellulase action.

FUTURE DIRECTION

The cellulase enzyme production in A. cellulolyticus strain 11B has, to date, not been fully optimized. All fermentation runs were directed toward either enzyme production for enzyme purification purposes or improvements in the growth rate and resultant densities of the microorganism. Improved cellulase enzyme productivities will be studied by rigorous examination of growth conditions and inducer additions. Also, in the future, enzyme fractionation procedures will be carried to the point at which purity and the activities and characteristics of individual components will have been evaluated (estimated close date, December, 1986).

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III. ION-MODERATED PARTITION CHROMATOGRAPHY (IMP) AT LOW TEMPERATURE: APPLICATION TO ANOMERIC CONFIGURATIONAL ANALYSIS OF MONOSACCHARIDES AND DISACCHARIDES LIBERATED BY ENZYMATIC HYDROLYSIS

John O. Baker and Michael E. Himmel

ABSTRACT

Analytically useful separations of the α- and β-anomers of five economically important monosaccharides (glucose, xylose, galactose, mannose and arabinose) can be obtained by totally aqueous chromatography at 1.5°C on a commercially-available calcium-form ion-exchange column, the Bio-Rad HPX-87C. Such analyses are expected to be very important in determining the mechanisms of action of different enzymes converting the polysaccharides found in woody biomass to monomeric units fermentable to fuel alcohol. Aqueous chromatography on a similar column having a different metal counterion, the lead-form Bio-Rad HPX-87P, separates the anomers of glucose but fails to separate the anomers of the other four sugars that are anomerically resolved by the calcium column. This counterion-dependence is shown to be due not to a lack of chromatographic selectivity by the lead-form column, but to the substantially higher rate of mutarotation of the sugars in the presence of the lead-form packing material. Analysis of the shapes of the elution profiles yields estimates of the effective rate constants for mutarotation of glucose on the calcium columns; the observed perturbation of the values of k_α and k_β with respect to those measured in water at the same temperature in turn suggest a possible mechanism for the anomeric separation. Both the theoretical and applied aspects of this work have been detailed recently in the professional literature

[1]; some of the more important applied aspects are briefly discussed below.

INTRODUCTION

The α- and β-anomers of monosaccharides have been shown to be separable by chromatography at low temperature on ion-exchange resins--using either aqueous-organic eluent systems as first reported by Ramnas and Samuelson [2] in 1974, or pure water as eluent as described by Goulding [3] in 1975. The systems using aqueous-organic mixtures as eluent [2,4-8] are, in general, capable of somewhat better resolution than are the systems using pure water, due to the decreased rate of mutarotation in systems having decreased concentrations of water [9], and especially if advantage is taken of the antifreeze properties of such systems in order to operate below the freezing point of water [2]. For certain applications, however, there are good reasons for preferring purely aqueous systems. One such application is the determination of the anomeric configuration of the products resulting from enzymatic hydrolysis of glycosidic bonds--for instance, the conversion of polymers such as starch and cellulose to monomeric glucose [10-14]. In such determinations, it is desired that a relatively high concentration of product be generated before any sizeable percentage of the product has changed its anomeric configuration through mutarotation. To this end, rather high concentrations of enzyme are used, in order to provide rates of generation of product much greater than the rate of subsequent mutarotation of the product in solution. If aliquots from such reaction mixtures are injected directly onto a chromatographic system using an aqueous-organic mixture as eluent, there is a high probability that the protein will precipitate on the column. Procedures are available for avoiding such an undesirable result by removal of protein from the sample prior to chromatography, but in view of the desirability of speed and operational simplicity in such determinations and also the desirability of avoiding uncertainties concerning the quantitative transfer of sugars in extraction steps, a chromatographic procedure allowing direct injection of reaction-mixture aliquots is distinctly advantageous. We have shown [1] that several commercially available HPLC columns having calcium- or lead-form sulfonated polystyrene packing materials are capable of separating sugar anomers efficiently if operated at 1.5°C with pure water as eluent, thus permitting direct injection of reaction-mixture aliquots containing substantial amounts of protein.

EXPERIMENTAL

All columns used in this study (HPX-87C, HPX-87P, and HPX-42C, each 300 x 7.8 mm) were purchased from Bio-Rad, and were maintained at temperatures from 72°C down to 1.5°C by means of a water jacket connected to a circulating water bath. In all cases the flow rate was 0.3 mL of deionized water per minute, and the injection volume was 20 µL. Detection was by a high sensitivity Hewlett-Packard R.I. detector (Erma Instruments, Japan). The enzyme substrates salicin and p-nitrophenyl-α-D-glucopyranoside, as well as the enzymes almond β-glucosidase (β-D-glucoside glucohydrolase; EC 2.1.1.21) and yeast α-
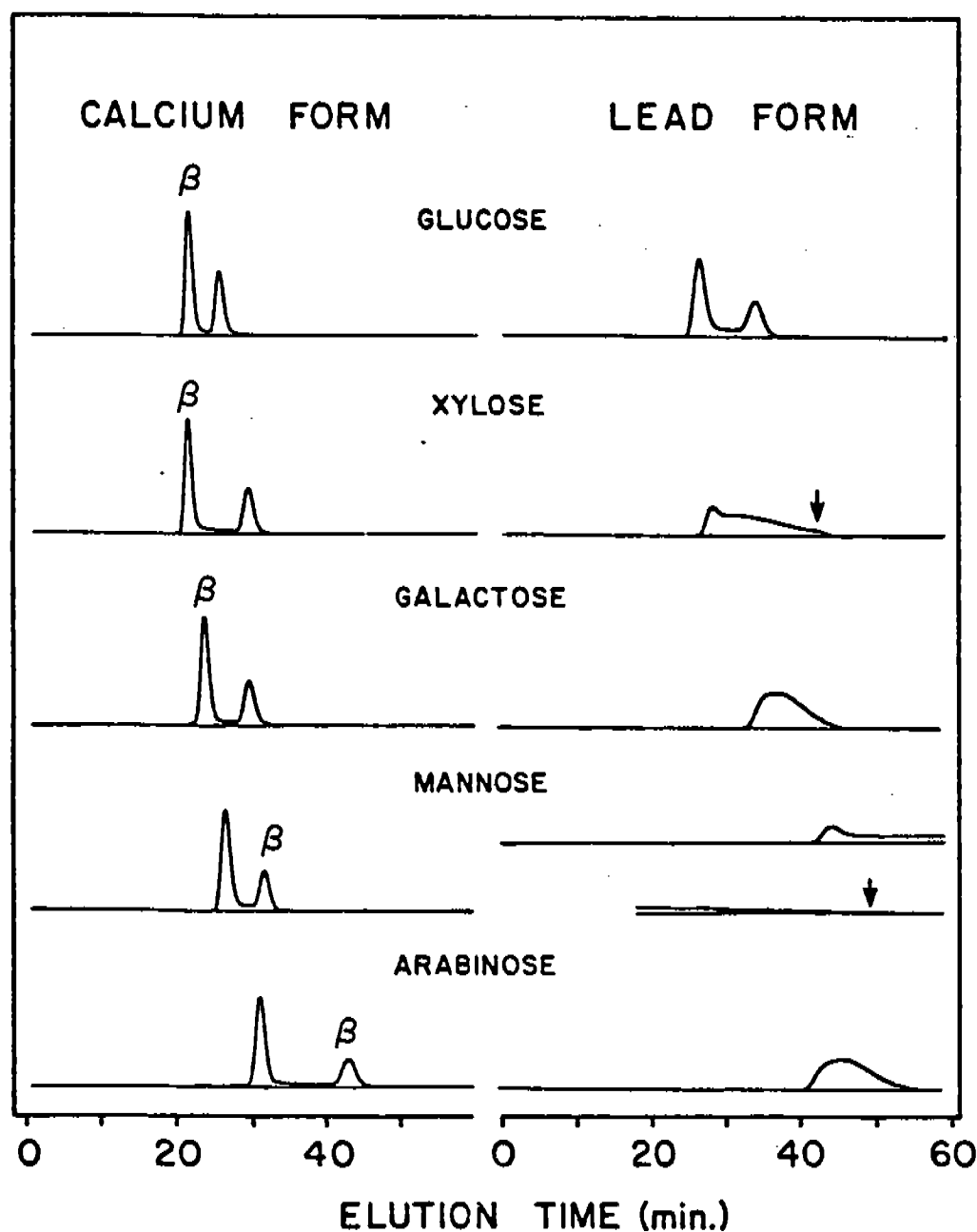
Figure 1. Separation of sugar anomers by aqueous chromatography on calcium- and lead-form ion-exchange columns. Chromatograms on the left were run on the calcium-form Bio-Rad HPX-87C, those on the right, on the lead-form Bio-Rad HPX-87P. For both columns, operating temperature was 1.5°C; flow rate was 0.3 mL/min. Sample size: 100 μg in 0.02 mL, detection by refractive-index deflection.

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glucosidase (α-D-glucoside glucohydrolase; EC 3.2.1.20) were purchased from Sigma.

RESULTS

Analytically useful separations of the α- and β-anomers of five economically important monosaccharide building blocks of woody biomass (glucose, xylose, galactose, mannose and arabinose) can be obtained by totally aqueous chromatography at 1.5°C on a commercially-available calcium-form ion-exchange column, the Bio-Rad HPX-87C, whereas aqueous chromatography on a similar column having a different metal counterion, the lead-form Bio-Rad HPX-87P, separates the anomers of glucose but fails to separate the anomers of the other four sugars that are anomerically resolved by the calcium column (see Figure 1).

Application of these separations to determination of the product stereospecificity of glycohydrolase reactions is shown in Figure 2 (A,B). The preponderance of the β-anomer in the glucose split from salicin by almond β-glucosidase, and the preponderance of α-anomer in the product split from p-nitrophenyl-α-D-glucopyranoside by brewer's yeast α-glucosidase, are dramatically demonstrated.

A third column of this general type, the calcium-form HPX-42C, has a more open bead structure than the other two columns and is capable of useful separations of the anomers of the disaccharides cellobiose and maltose (Figure 3). The HPX-42C therefore promises to be useful in determining the anomeric configuration of the products of enzymes, such as β-amylase (1,4-α-D-glucan maltohydrolase; EC 3.2.1.2) and 1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91, that act on polysaccharides to liberate disaccharides rather than monosaccharides.

DISCUSSION

The method of anomeric analysis described here has a number of advantages with respect to other methods. The capability of injecting reaction-mixture aliquots directly without any need for prior derivatization or extraction steps facilitates the examination of early stages of enzyme reactions. Since the aqueous metal-form ion-exchange method measures specific chemical species, rather than additive properties of solutions such as optical rotation, the LC method will be less suceptible to certain interferences. The sensitivity of the method, allowing determinations to be made on reaction mixtures of 100 μL or less, is certain to be welcomed by workers using limited and precious supplies of purified enzymes. Finally, of some practical advantage is the fact that many of the laboratories concerned with mechanistic studies of enzymes hydrolyzing glucosidic bonds will already have these columns, or their equivalents, on hand for use at high temperatures in routine assay of enzyme activity. Implementation of the procedure described here will, therefore, involve only the provision of means for maintaining the columns at low temperatures.

FUTURE DIRECTION

The methodology discussed above will be applied to classification of new

hydrolytic enzymes encountered in the future, as well as those currently under study. The development work on the analytical problem itself is now closed, however.

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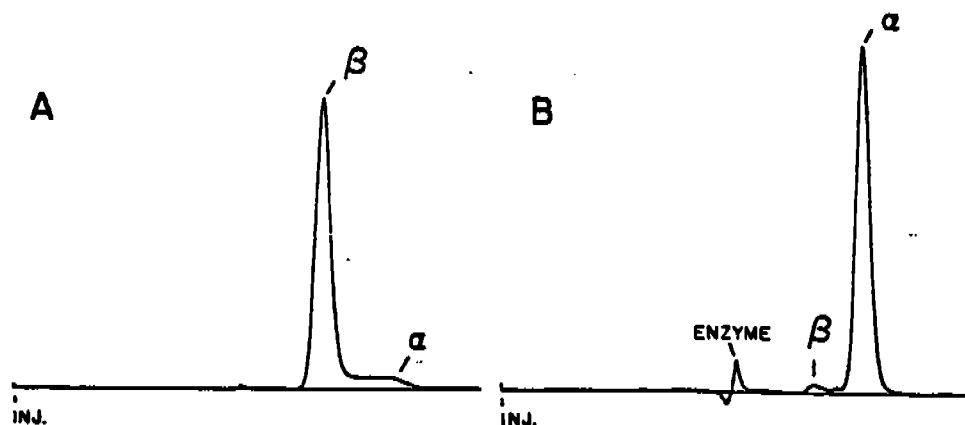
ACKNOWLEDGMENTS

Figure 2. Determination of anomeric product specificity for enzymic cleavage of synthetic substrates. A: Chromatography on lead-form column (HPX-87P) of the products of a 2.5-min. incubation at 22°C of 4.0 mg/mL salicin [2-(hydroxymethyl) phenyl-β-D-glucopyranoside] with 0.67 mg/mL almond β-glucosidase, at pH 5.0 in 3.33 mM acetate. B: Chromatography on calcium-form column of the products of a 2.5-min incubation of p-nitrophenyl-α-D-glucopyranoside (2.5 mg/mL) with 1.25 mg/mL brewer's yeast α-glucosidase at 22°C, pH 5.0 in 2.5 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer.
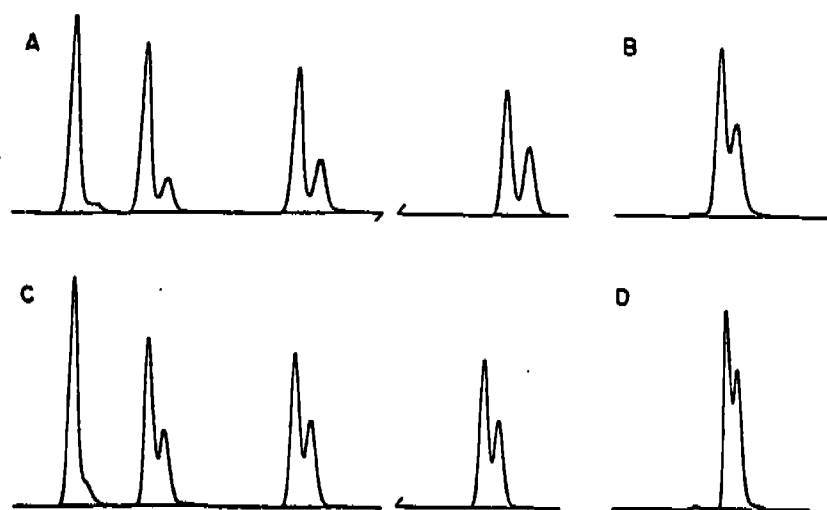

Figure 3. Separation of the anomers of disaccharides on calcium-form ion-exchange columns. Injection volume 0.020 mL for all samples; column temperature 1.5°C, eluent deionized water at 0.15 mL/min. (A) Sequential injections on HPX-42C of 5.0-mg/mL maltose at (from left to right) 70 seconds, 20 min, 63 min, and 29 hours after time of solution in water at 22°. (B) equil-maltose, 5.0 mg/mL, chromatographed on HPX-87C (C) Cellobiose, 1.0 mg/mL, injected onto HPX-42C at (left to right) 2 min, 110 min, 4 hrs 10 min, and 31 hrs after time of solution in water (D) equil-cellobiose chromatographed on HPX-87C.

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